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2. Patent application number *(The Patent Office will fill this part in)*	0402106.9	30 JAN 2004
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	SYNGENTA PARTICIPATIONS AG Intellectual Property Department Schwarzwaldallee 215 4058 Basel SWITZERLAND	
Patents ADP number *(if you know it)*	8029555001	
If the applicant is a corporate body, give the country/state of its incorporation		
4. Title of the invention	**IMPROVED FERTILITY RESTORATION FOR OGURA CYTOPLASMIC MALE STERILE *BRASSICA* AND METHOD**	
5. Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**Jozsef Christopher GAAL** Intellectual Property Department Syngenta Limited Jealott's Hill International Research Centre PO Box 3538 Bracknell, Berkshire, RG42 6YA UNITED KINGDOM	
Patents ADP number *(if you know it)*	8029563001	

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Description	18 + 28 pages of sequence listing
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Request for a preliminary examination and search (Patents Form 9/77)	One
Request for a substantive examination (Patents Form 10/77)	
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Signature(s) Anne Tarry   Date 30.01.04.

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom   Anne TARRY – 01344 413616

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IMPROVED FERTILITY RESTORATION FOR OGURA CYTOPLASMIC MALE STERILE *BRASSICA* AND METHOD

FIELD OF THE INVENTION

This invention relates to a novel fertility restorer locus for Ogura cytoplasmic male sterile *Brassica* plants.

BACKGROUND OF THE INVENTION

Oilseed rape (*Brassica napus*), also referred to as canola (annual spring type) or winter oilseed rape (biannual type), is derived from interspecific hybridization of *B. oleracea* and *B. campestris*. Breeding between *Brassica* species is common. Generally, winter-type rapeseed is grown in North Western Europe, whereas spring-types are grown in Canada, China, India, Australia and South America mainly.

Oilseed rape is becoming an increasingly important crop, valued for edible and industrial oil usage and for its seed meal rich in protein. Wide acceptance of rapeseed meal for animal nutrition is hampered by the presence in the seed of sulfur compounds called glucosinolates (GSLs). Glucosinolates are undesirable in Brassica seeds since they can lead to the production of antinutritional breakdown products upon enzymatic cleavage during oil extraction and digestion. Although the development of superior, edible oil had been achieved in the early 1970s through introduction of rapeseed varieties with less than 2% of erucic acid in percent of their total fatty acid profile (single zero quality), the continuing presence of glucosinolates in the high protein meal remained a major constraint to further market expansion.

At present the maximum threshold for GSL free rapeseed varieties set by European law is 25μmol total glucosinolate (GSL) per gram (g) of seed at 9% humidity (EU decree 2294/92). Doublelow spring canola varieties cultivated in Canada need to have GSL levels of less than 30μmoles of glucosinolates per gram of air-dried oil-free meal. The GSL levels of commonly cultivated double zero oilseed rape varieties in Europe and Canada varies significantly below the threshold levels at 60% of the official threshold level or even lower. At present, hybrid *Brassica* plants based on the Ogura hybrid system having seeds with low

GSL content express inferior agronomical traits such as lower seed yields, poor disease resistances and lodging susceptibility.

Hybrid cultivars are desired because of potentially higher seed yield due to heterosis. To produce hybrid *Brassica* plants, breeders use self-incompatible (SI), cytoplasmic male sterile (CMS), or nuclear male sterile (NMS) *Brassica* plants as the female parent. SI plants are not able to self pollinate due to their genetic constitution and CMS and NMS female plants are incapable of producing pollen. Thus, all these plants must be cross-pollinated by a male parent. A number of CMS systems are used for hybrid seed production of *Brassica*: Polima (*pol*), *nap*, *tournefortii*, Kosena, and Ogura (*ogu*). (See for example Ogura (1968) Mem. Fac. Agric. Kagoshima Univ. 6:39-78; Makaroff (1989 Journal of Biol. Chem. 264: 11706-11713; US Pat. No. 5,254,802.) The *ogu* system, thought to be the most useful, is based on the use of a male sterility determinant derived from *Raphanus sativus* cytoplasm. F1 seed produced from a cross between an Ogura CMS female *Brassica* plant and a normal male *Brassica* plant will be male sterile. In other words, plants grown from the F1 seed will not produce pollen. To produce a male fertile F1 generation plant, a restorer gene must be present in the male parent of the F1 hybrid.

A fertility restorer locus was transferred from *Raphanus sativus* to *Brassica* CMS plants by Institut National de la Recherche Agronomique (INRA) in Rennes, France (Pelletier et al., 1987) Proc 7th. Int. Rapeseed Conf., Poznan, Poland: 113-119. The restorer gene (Rf) originating from *Raphanus sativus* is described in WO92/05251 and in Delourme et al., (1991) Proc. 8th. Int. Rapeseed Conf. Saskatoon, Canada: 1506-1510. The original restorer inbreds and hybrids carrying this Rf gene express elevated glucosinolate levels and a decrease in seed set (Pellan-Delourme and Renard, 1988 Genome 30: 234-238, Delourme et al., 1994 Theor. Appl. Genet. 88: 741-748). In seed grown on Ogu Rf hybrid plants, the glucosinolate levels are elevated even when the female parent has reduced glucosinolate content. Recombination at the radish chromosomal region surrounding the Rf gene is suppressed in *Brassica* and therefore different recombination events in this region are difficult to obtain. The link between the Rf gene and the glucosinolate locus has been broken (WO98/27806). However, it is difficult to break the linkage between the glucosinolate gene and the restorer gene and still maintain line stability and superior combining ability for the production of high value commercial hybrid seed. Therefore, there is a need to develop a recombination event

that unlinks the restorer gene from the glucosinolate gene while maintaining a *Brassica* plant's ability to produce high value commercial hybrid seed.

SUMMARY OF THE INVENTION

The current invention provides a *Brassica* plant that comprises a recombination event resulting from a break between the restorer locus and the glucosinolate locus along a nucleic acid segment derived from the ogura *Raphanus sativus* and subsequent rejoining to produce a new nucleic acid segment, referred to herein as the BLR1 recombination event.

A *Brassica* plant of the present invention expresses fertility restoring resulting from expression of the *Raphanus sativus* restorer gene and a GSL content no higher than normal doublelow open pollinated varieties (varieties low in erucic acid in the oil and low in GSL in the solid meal remaining after oil extraction). The *Brassica* inbred line BLR-038, Deposit Number NCIMB-41193, is one example of a plant that contains the BLR1 recombination event of the invention. Using breeding techniques known to those skilled in the art and as briefly described herein, inbred line BLR-038 and other plants containing the BLR1 recombination event of the invention are crossed with male sterile inbreds to produce hybrids expressing low GSL content and superior agronomic traits. More generally, the present invention also includes transferring the BLR1 recombination event of the present invention from one *Brassica* plant to another. The present invention further includes the use of marker-assisted selection to select *Brassica* plants containing the BLR1 recombination event.

The invention includes a *Brassica* plant comprising a *Raphanus sativus* DNA fragment including a restorer gene, wherein said DNA fragment hybridizes to at least one marker of bin 2, but not to a marker of bin 3, and wherein the DNA fragment is the BLR1 recombination event of the present invention.

The present invention includes markers of bin 2 comprising E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2, and markers of bin 3 comprising OPY17, OPN20, and E8M1-2.

The present invention further includes the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2 and E8M14-2, which are amplified in a polymerase chain reaction using primer pairs represented by 1159 and 1160; E2 and M4; E3 and M1; E4 and M14; E5 and M1; and E8 and M14, respectively.

The present invention also includes the markers OPY17, OPN20, and E8M1-2, which are amplified in a polymerase chain reaction using the primer pairs represented by PR0004F and PR0004R; 1135 and 1136; and E8 and M1.

The invention includes the BLR1 recombination event, which is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

The present invention includes a method of detecting a *Brassica* plant containing a restorer gene derived from *Raphanus sativus*, comprising the steps of: obtaining a plant sample from a *Brassica* plant, detecting in the sample a DNA fragment that can be detected by a marker of bin 2, but not by a marker of bin 3. The method further includes selecting the *Brassica* plant, or a part thereof, containing the DNA fragment, and also selfing the *Brassica* plant containing the DNA fragment. Preferably, the DNA fragment comprises the BLR1 recombination event.

The present invention further includes a method of detecting a *Brassica* plant, wherein the marker of bin 2 comprises E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

The invention includes a method of detecting a *Brassica* plant, wherein the marker of bin 2 has partial homology to E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

The method of the invention includes the step of detecting in a plant sample a DNA fragment obtainable by PCR amplification using primers 1159 and 1160, whereas the DNA fragment is not amplified by the primers PR0004F and PR0004R.

The present invention also includes a combination of markers for detecting the presence of the BLR1 recombination event, comprising a marker of bin 2 and a marker of bin 3.

The present invention further includes a marker of bin 2 comprising E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2 and a marker of bin 3 comprising OPY17, OPN20, and E8M1, or a marker having partial homology to any one of these markers.

The present invention also includes a method for screening a *Brassica* plant to determine whether it contains the BLR1 recombination event, comprising extracting DNA from the *Brassica* plant, subjecting the *Brassica* plant extraction to a polymerase chain amplification

reaction in the presence of primers 1159, 1160, PR0004F, PR0004R, and determining the amplification of DNA fragments from the extracted DNA by primers 1159 and 1160 and lack of amplification of DNA fragments from extracted DNA by primers PR0004F and PR0004R, thereby indicating the presence of the BLR1 recombination event.

The present invention also includes a method for producing a fertile F1 hybrid *Brassica* plant comprising the steps of crossing a Brassica male fertile plant comprising the BLR1 recombination event with a *Brassica* CMS male sterile plant to produce F1 fertile seed, further comprising the step of planting said F1 hybrid seed, and further comprising the step of harvesting the F2 seed grown from the plant resulting from said F1 seed, and includes F1 hybrid *Brassica* plants developed by this method.

The present invention also includes a *Brassica* plant comprising the BLR1 recombination event, wherein said event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

The present invention further includes a method of introgressing the BLR1 recombination event comprising the steps of obtaining a *Brassica* plant containing the BLR1 recombination event, crossing this plant with another *Brassica* plant, producing hybrid seed and selecting hybrid seed containing the BLR1 recombination event.

The present invention includes a kit and method that incorporate one or more of markers falling within bin 2 and one or more markers falling within bin 3 to detect the presence of the BLR1 recombination event in a plant or a plant part. According to the invention, plant material that contains the BLR1 recombination event hybridizes to bin 2 markers, but not to markers of bin 3.

BRIEF DESCRIPTION OF THE FIGURES

FIG 1 is a photocopy of an agarose gel illustrating amplification products that are generated and analyzed by the multiplex PCR kit and method of the present invention. Lane 1: random maintainer genotype rr; 2: Pioneer 209002; 3: BLR 038; 4: Pioneer 97838, 5: Pioneer 97839; 6: Pioneer 209001; 7: original *Ogura* restorer Rr, 8: Lutin (INRA) a *Raphanus sativus* restorer gene.

FIG. 2 is a schematic depiction of the F1 hybrid production scheme for CMS *Brassica* plants.

DETAILED DESCRIPTION OF THE INVENTION

The current invention provides a *Brassica* plant comprising a unique recombination event, referred to herein as the BLR1 recombination event, due to a break at a position along the radish nucleic acid segment between the restorer locus and the glucosinolate locus. *Brassica* plants of the present invention express fertility restoring resulting from expression of the *Raphanus sativus* restorer gene and a GSL content no higher than normal double low open pollinated varieties. The *Brassica* inbred line BLR-038, Deposit Number NCIMB-41193, is one example of a plant that contains the BLR1 recombination event. Using breeding techniques known to those skilled in the art and as briefly described herein, inbred line BLR-038 and other inbred lines containing the BLR1 recombination event are crossed with male sterile inbreds to produce hybrids expressing low GSL content and superior agronomic traits. More generally, the present invention further includes transferring the BLR1 recombination event of the present invention from one *Brassica* plant to another. A further aspect of the invention is a kit and method including markers and the use of markers of specified bins to select *Brassica* plants that contain the BLR1 recombination event.

Breeding History of the *Brassica* inbred line BLR038 and GSL characterization

Table 1 describes the breeding history of plants of the present invention containing the BLR1 recombination event, which is a recombination of the ogura *Raphanus sativus* restorer locus. In year 1992, the CMS inbred line R30915 was crossed with the male inbred line R40 containing the restorer gene of INRA, to produce F1 hybrids. R40 is a generation F6 offspring produced via selfings from the original cross (Fu 58.Darmor B1F1 x Rest. Darmor B1F1) x Bienvenu. F1 hybrids from the cross R30915 x R40 with the CMS-restorer gene were selected based on male fertility, which was determined at flowering. The F1 hybrid plants (92HR013) were crossed with a non-CMS, non-restorer double zero quality breeding line 93B-1-3. In 1994, seeds of fertile plants resulting from the cross with 93B-1-3 were grown and the resulting CMS restorer plants were crossed with the double low quality breeding line 92/19047. The lines resulting from this cross were selfed several times from 1995 through 2002 as shown in Table 1. In all plots, segregation of male fertility was

observed, meaning that all plots contained heterozygous and homozygous maintainer and restorer plants. Because all crosses were initially made in the Ogura CMS cytoplasm and this cytoplasm was maintained in all future generations the maintainer genotypes turned out to be male sterile. Plants were selfed using plastic bags to cover the inflorescence before flowering. The bag was maintained over the plant during the whole flowering period to avoid cross-pollination.

The GSL content of the *Brassica* seeds was monitored throughout the development of inbred line BLR-038. Glucosinolate content is given in µmol/g of seed at 9% humidity. The glucosinolate analysis was performed using the near-infrared reflectance spectroscopy. Using this method, it is possible to analyze samples of undestroyed *Brassica* seed on their quality components oil, protein and glucosinolate. The analyses were performed on a FOSS NIR Systems Model 5000-c. Glucosinolate analysis is described in P. Williams and D. Sobering, (1992) In: Hildrum K., Isaksson T., Naes T. and Tandberg A. (eds.) Near Infra-red Spectroscopy. Bridging the gap between Data Analysis and NIR Applications. Horwood Chichester, UK: 41-446

In 1999, one plant of the F6 generation, 22044-3, had a GSL content of 17.3 µmol/g seed, while the seed of its sister plants had a GSL content between 22.5-23.8 µmol/g. Plant 22044-3 was selfed resulting in plants of the F7 generation. Seed of the 6797-2 plant had a GSL content of 11.4 µmol/g, while its sister plants had a GSL content from 24.6-25.7 umol/g. The plant resulting from growing the seed of 6797-2 was selfed. In 2001 at F8, no single plant resulting from this selfing had seed with a GSL content above 14.3 umol/g. The seed of plant 21615-7 had a GSL content of only 7.0 umol/g. The average expression of seed from plants in plot 21615 was 10.7 umol/g, which is at least 7 µmol lower than the lowest other reference restorer lines grown simultaneously in the same experimental field trial in Germany and more than 5 µmol below the standard plots of the non-restorer varieties Express and Laser. At the F9 generation, BLR-038 was produced by selfing of homozygous descendants of 21615-5.

TABLE 1

Pollination	Year	Generation	PLOT	Cross	Plot µmol/g Seed	Single plant No. (GSL µmol/g seed at 9% H_2O)
cross	1992		92HR013	R30195 (CMS B6 021) x R40		
cross	1993		93HR141	92HR013 x 93B-1-3		
cross	1994	F1	94HR233	93HR141 X 92/19047		
selfing	1995	F2	21614			9
selfing	1996	F3	21969			3
selfing	1997	F4	22446			8
selfing	1998	F5	22590			1
selfing	1999	F6	22044	GSL content of sister plants was 22.5-23.8		3(17.3)
selfing	2000	F7	6797	GSL content of sister plants was 24.6-25.7		2 (11.4)
selfing	2001	F8	21615	No single plants with GSL content above 14.3µmol were observed	10.7	1 (10.3), 2 (9.4), 4 (14.1), 5 (8.6), 6 (9.4), 7 (7.0), 8 (14.3)
selfing	2002	F9	21615-5			BLR-038

Table 2 shows the segregation ratio for several of the single plants of plot 01-21615. The Rf pollinator plants (21615-01, 21615-05, 21615-06, 21615-08) are homozygous for the Rf gene (RfRf). F1 hybrids were produced from the cross of the homozygous Rf pollinator and CMS female lines. These crosses show a transmission of male fertility of approximately 100%.

TABLE 2

	Homozygous Pollinator							
	F1 hybrids				Selfing of Pollinator			
Origin 2001 (F8)	plants	male sterile	male fertile	ratio fertile plants; expected 100%	plants	male sterile	male fertile	ratio fertile plants; expected 100%
01 21615-01	12	0	12	100.0%	9	0	9	100.0%
01 21615-05	39	1	38	97.4%	14	1	13	92.9%
01 21615-06	16	0	16	100.0%	12	0	12	100.0%
01 21615-08	11	1	10	90.9%	9	0	9	100.0%
SUM	78	2	76	97.4%	44	2	43	97.7%

Characterization of *Brassica* inbred line BLR-038 by means of AFLP analysis

A population consisting of 25 individuals segregating for the original Ogura restorer translocation was genotyped using a co-dominant PCR assay consisting of two proprietary SCAR markers derived from RAPD marker OPY17 that are in coupling or in repulsion phase to the restoration locus. Homozygous recessive (rf/rf) plants and restorer (RfRf and Rfrf) plants were bulked separately and used for the identification of AFLP markers putatively linked to the Rf gene. Such markers allowed for the comparison of BLR-038 to Pioneer hybrids 209002, 97839, 97838, 209001, and to the SERASEM hybrid Lutin containing the restorer locus released by INRA. AFLP analysis was performed essentially as described by Vos et al. (1995) Nucleic Acids Research 23(21): 4407-4414.

First, 500 ng DNA for each sample BLR-038, 209002, 97839, 97838, 209001, and the hybrid Lutin, was digested in 40µl of 1×TA-buffer (10 mM Tris-acetate, 10 mM MgAc, 50 mM KAc, 1 mM DTT, 2 µg BSA and 5 u each of *Eco*RI and *Tru*1I (MBI Fermentas, Lithuania). *Eco*RI is in the following referred to as E, and *Tru*1I, an isoschizomer of *Mse*I, is referred to as M. The E and M adaptors are represented by the following sequences:

EcoRI-adaptor:	5'-CTCGTAGACTGCGTACC	SEQ ID NO: 21
	CATCTGACGCATGGTTAA-5'	SEQ ID NO: 22
MseI-adaptor:	5'-GACGATGAGTCCTGAG	SEQ ID NO: 23
	TACTCAGGACTCAT-5'	SEQ ID NO: 24

Following digestion, 10 μl of ligation solution containing 1×Ligation buffer (40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 1 u T4 DNA ligase, 0.1 μM E-adapter and 1.0 μM M-adapter, sequences as described by Vos et al. (1995), was added directly to the DNA digest, incubated, and subsequently diluted 10-fold in 1×TE-buffer. To increase the amount of template DNA, the diluted ligation reactions were preamplified with primers having one additional and selective nucleotide each, i.e. E+1 and M+1, The primers used for the pre-amplification reaction consist of the same sequence as the adapters except for a one nucleotide extension at their 3' end. Primer E+A hybridizes to the EcoRI adapter and carries an additional A, the primer M+C hybridizes to the MseI adapter and carries an additional C. The reaction solution of 20 μl contained 5 μl of template DNA (10-fold diluted ligation reaction), 1×PCR-buffer II (10 mM Tris-HCl,pH 8.3), 50 mM KCl, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.4 u *Taq* polymerase and 0.3 μM each of (E+A)-primer and (M+C)-primer. The pre-ampflication reactions were performed in either Perkin-Elmer/Cetus 9600 or MJ Research PTC-100 thermocyclers using the following temperature profile: 20 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C.

Prior to selective amplification, (E+3)-primers were end-labelled in a solution containing 1×kinase buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 1.7 μM (E+3)-primer (DNA Technologies), 0.2 u/μl T4 polynucleotide kinase and 2 μCi/μl μ-^{33}P[ATP]. Selective amplification was performed using the following temperature profile: 12 cycles of 30 s at 94°C, 30 s at 65°C ramping 0.7°C/cycle to 56°C, 60 s at 72°C, followed by 23 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C. The reaction solution of 20 μl contained 5 μl pre-amplified template DNA, 0.5 μl labelled (E+3)-primer, 1×PCR-buffer II (Advanced Biotechnologies), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μM (M+3)-primer (DNA Technologies), and 0.4 u of *Taq* polymerase. After amplification 20 μl of formamide loading buffer (98% formamide, 10 mM EDTA, 0.1% each of xylene cyanol and bromophenol blue) was added and the samples were denatured at 95°C

for 3 min. Amplified fragments were separated on 5% polyacryamide gels consisting of 19:1 Acrylamide/Bis solution, 1×TBE-buffer, 0.10% TEMED and 0.03% APS. Custom-made gel apparatuses for 35 cm gels (CBS Scientific Co., USA) were used in all analyses. Gels were pre-run at 110 W for 30 min before loading of 3 µl sample and run at 110W for 3 h. Following electrophoresis, gels were transferred to 3MM-paper, dried on a gel dryer over night at 80°C, and exposed to film for 1-2 days.

All E+3 primers (24 nt in length) as shown in Table 3 (SEQ. ID No. 25 to 37) and the sequence listing carry an A at position 22 and all M+3 primers (21 nt in length) a C at position 19, which correspond to the extensions on the pre-amplification primers. The extensions at the pre-amplification primers are random and are added for the purpose of reducing the complexity of the template. Rather than amplifying the whole genome, only a fraction is amplified that subsequently is used as template in the final amplification using the E+3 and M+3 primers. The E+A and M+C pre-amplification primers are identical to the E+3 and M+3 primers respectively, but two nucleotides shorter. It is understood that one skilled in the art can develop additional primers by generating additional randomly generated extensions to the adaptors M and E. Some of these new primers would amplify additional nucleic acid segments or markers located along the nucleic acid segment derived from ogura *Raphanus sativus* and would be categorized within one of the four bins. Those skilled in the art would recognize that these additional primers and markers fall within the scope of the claimed invention.

In total 48 primer combinations were screened, including the 7 primer pairs that were shown to deliver polymorphic bands in patent application WO98/56948. Only bands that were present in the Ogura Rf bulk but absent in the homozygous recessive bulk (rf/rf) were taken into consideration for the comparison of the *Brassica* inbred line BLR-038 to the hybrids released by Pioneer and INRA.

Table 3 shows all AFLP markers that revealed polymorphism between the bulk for the Ogura Rf translocation and the homozygous recessive (rfrf) bulk. The markers are binned according to their amplification profile across the various plant materials. The results are represented in a schematic manner in Table 4, which reveals the four different classes of markers. Presence of a band is indicated with '1', its absence with '0'. A bin refers to a set of markers grouped according to their location along a nucleic acid segment. AFLP markers

E5M16-1, E5M4-3, E6M3-2, and E8M14-1 are of bin 1, wherein these markers are amplified in all samples Lutin, P209001, P97838, P97839, BLR-038, and P209002. ALFP markers E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, E8M14-2 are of bin 2, wherein bin 2 markers amplify Lutin, P209001, P97838, P97839, BLR-038, but not P209002. The AFLP marker E8M1-2 is of bin 3, wherein bin 3 markers amplify Lutin, P209001, P97838, P97839, but not BLR-038, and P209002. The AFLP markers E2M13-1, E2M14-1, E3M12-1, and E6M3-1 are of bin 4, wherein bin 4 markers amplify Lutin and P209001, but not P97838, P97839, BLR-038, and P209002.

Characterization of the *Brassica* inbred line BLR-038 using SCAR markers

Primer pairs were designed to the nucleotide sequences of the amplification products for the RAPD, AFLP and SCAR markers in coupling phase with the Ogura restorer gene as disclosed in patent application CA2,206,673: OPC2 (Seq ID No. 2 and 7), OPN20 (Seq ID No. 3 and 8), OPF10 (Seq ID No. 4 and 10), OPH3 (Seq ID No: 9); OPH15 (Seq ID No. 11); E36xM48AIII ((Seq ID No. 12), E35xM62AV (Seq ID No. 13), E33xM47A1 (Seq ID No. 14), and E38xM60A1 (Seq ID No. 15). In addition to these markers, primers were designed to the nucleotide sequence of RAPD marker OPH11 that was shown to be associated to fertility restoration in *Raphanus* where the Ogura locus originates (Accession number AB051636). The sequences of all primers assayed as well as the size of the expected amplification products are listed in Table 3. The primer combinations including the proprietary SCAR marker derived from RAPD marker OPY17, were used to analyze the original Ogura translocation, BLR038, Pioneer hybrids 209002, 97839, 97838, 209001, and the hybrid Lutin using a standard PCR protocol. After PCR, the amplification products were visualized by means of agarose gel electrophoresis. Referring to Table 4, the SCAR markers OPF10, OPC2 AND E35M62 are markers of Bin 1. Markers that fall within Bin 1, as discussed above, are characterized as amplifying the samples Lutin, P209001, P97838, P97839, BLR-038, and P209002. The SCAR marker E33M47 is of bin 2. Bin 2 markers are characterized as amplifying the samples Lutin, P209001, P97838, P97839, BLR-038, but not P209002. The two SCAR markers, OPY17 and OPN20 of Bin 3, are characterized by amplifying the samples Lutin, P209001, P97838, P97839, but not BLR-038, and P209002. Bin 4 SCAR markers, such as OPH15 and E36M48, amplify Lutin and P209001, but not P97838, P97839, BLR-038, and P209002.

TABLE 3

Marker Locus	Primer Pair	Sequence	Product Size	Origin of sequence
SCAR markers and primers				
OPC2	1127 (SEQ ID NO: 1) 1128 (SEQ ID NO: 2)	ggggaaggaaggaaggactc tcaggttcacacagcagcata	677 bp	CA 2,206,673
OPN20	1135 (SEQ ID NO: 3) 1136 (SEQ ID NO: 4)	ataggttcctggcagagatg atagcagtcagaaaccgctc	630 bp	CA 2,206,673
OPF10	1137 (SEQ ID NO: 5) 1138 (SEQ ID NO: 6)	ctgatgaatctcggtgagac ccgtatgccttggttatctc	760 bp	CA 2,206,673
OPH15	1218 (SEQ ID NO: 7) 1219 (SEQ ID NO: 8)	tctgtaaatcctttccaccc aaaaaagcacccgagaatct	601 bp	CA 2,206,673
E36M48	1222 (SEQ ID NO: 9) 1223 (SEQ ID NO: 10)	gcgtgatgatctgttgagaa ggatttgtgggattggaaa	251 bp	CA 2,206,673
E35M62	1224 (SEQ ID NO: 11) 1225 (SEQ ID NO: 12)	gaggttcaggaatgctgttt gctcctgttagtgactcttca	201 bp	CA 2,206,673
E33M47	1159 (SEQ ID NO: 13) 1160 (SEQ ID NO: 14)	taacaaaatagagggagaggatg caagattatagctacctaacagg	140 bp	CA 2,206,673
Gene 16	16-1 (SEQ ID NO: 15) 16-2 (SEQ ID NO: 16)	tgttcagcatttagtttcgccc ttgttcagttccaccaccagcc	471 bp	WO 03/006622
Gene 26	26-1 (SEQ ID NO: 17) 26-2 (SEQ ID NO: 18)	gctcacctcatccatcttcctcag ctcgtcctttaccttctgtggttg	530 bp	WO 03/006622
OPY17	PR0004F (SEQ ID NO: 19) PR0004R (SEQ ID NO: 20)	acgtggtgaggacatgccctttctg ctggtgtattctacctcatcattaaa	300 bp	Syngenta
AFLP markers and primers				
E2M4	E2 (SEQ ID NO: 25) M4 (SEQ ID NO: 26)	ctcgtagactgcgtaccaattaac gacgatgagtcctgagtacat		
E2M13	E2 M13 (SEQ ID NO: 27)	 gacgatgagtcctgagtacta		
E2M14	E2 M14 (SEQ ID NO: 28)	 gacgatgagtcctgagtactc		

E3M1	E3 (SEQ ID NO: 29) M1 (SEQ ID NO: 30)	ctcgtagactgcgtaccaattaag gacgatgagtcctgagtacaa		
E3M12	E3 M12 (SEQ ID NO: 31)	 gacgatgagtcctgagtacgt		
E4M14	E4 (SEQ ID NO: 32) M14	ctcgtagactgcgtaccaattaat		
E5M1	E5 (SEQ ID NO: 33) M1	ctcgtagactgcgtaccaattaca		
E5M4	E5 M4			
E5M16	E5 M16 (SEQ ID NO: 34)	 gacgatgagtcctgagtactt		
E6M3	E6 (SEQ ID NO: 35) M3 (SEQ ID NO: 36)	ctcgtagactgcgtaccaattacc gacgatgagtcctgagtacag		
E8M1	E8 (SEQ ID NO: 37) M1	ctcgtagactgcgtaccaattact		
E8M14	E8 M14			

TABLE 4

Marker	Type	Rr	Rr	Lutin	P 209001	P 97838	P 97839	BLR-038	P 209002	
		1	2	8	6	4	5	3	7	
OPF10	SCAR	1	0	1	1	1	1	1	1	
OPC2	SCAR	1	0	1	1	1	1	1	1	
E35M62	SCAR	1	0	1	1	1	1	1	1	
E5M16-1	AFLP	1	0	1	1	1	1	1	1	BIN 1
E5M4-3	AFLP	1	0	1	1	1	1	1	1	
E6M3-2	AFLP	1	0	1	1	1	1	1	1	
E8M14-1	AFLP	1	0	1	1	1	1	1	1	
E33M47	SCAR	1	0	1	1	1	1	1	0	
E2M4-1	AFLP	1	0	1	1	1	1	1	0	
E3M1-1	AFLP	1	0	1	1	1	1	1	0	
E4M14-1	AFLP	1	0	1	1	1	1	1	0	BIN 2
E5M1-2	AFLP	1	0	1	1	1	1	1	0	
E5M4-2	AFLP	1	0	1	1	1	1	1	0	
E8M14-2	AFLP	1	0	1	1	1	1	1	0	
OPY17	SCAR	1	0	1	1	1	1	0	0	
OPN20	SCAR	1	0	1	1	1	1	0	0	BIN 3
E8M1-2	AFLP	1	0	1	1	1	1	0	0	
OPH15	SCAR	1	0	1	1	0	0	0	0	
E36M48	SCAR	1	0	1	1	0	0	0	0	
E2M13-1	AFLP	1	0	1	1	0	0	0	0	BIN 4
E2M14-1	AFLP	1	0	1	1	0	0	0	0	
E3M12-1	AFLP	1	0	1	1	0	0	0	0	
E6M3-1	AFLP	1	0	1	1	0	0	0	0	

Kit and method for detecting the BLR1 recombination event

Total DNA is isolated from approximately 1 cm^2 of *Brassica* leaf tissue by using the Wizard® Magnetic 96 DNA Plant System (Promega). In one embodiment, the Multiplex PCR kit and method of the present invention detects the presence or absence of PCR amplification products corresponding to OPY17 (Bin 3) and E33M47 (Bin 2).

The four primers PR0004F, PR0004R, 1159 and 1160 (Table 4) are added to a reaction mixture at a concentration of 7.5 pmol each. Except for the multiplex nature, the composition of the PCR reaction is standard in the art, using Platinum Taq polymerase from Invitrogen. Amplification conditions are as follows: 5 minutes of initial denaturation at 94°C were followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 90 seconds at 72°C. PCR amplification products were separated on 2.0 % agarose gels.

Fig. 1 illustrates the results of the PCR reaction, wherein the presence of the BLR1 recombination event is established when the primers amplify the 140 bp product that corresponds to E33M47, but does not amplify the 300 bp product that corresponds to OPY17. Fig. 1 also shows that the PCR reaction amplified both OPY17 and E33M47 for the original *Ogura* restorer translocation fragment as well as the derived recombination events Pioneer 97838, 97839, 209001, and the Lutin event from INRA. Pioneer recombination event 209002 on the other hand, shows neither the E33M47, nor the OPY17 amplification product. These results demonstrate that primers that selectively amplify markers from Bin 2 and 3, such as E33M47 and OPY17, are successfully used in a single multiplex PCR assay to distinguish and identify the BLR1 recombination event in plant material.

It is understood that the kit and method of the present invention incorporate one or more of markers falling within Bin 3 and one or more markers falling within Bin 2 to detect the presence of the BLR1 recombination event in plants. It is within the scope of the claimed invention to develop and use additional markers that fall within one of the bins 1, 2, 3, or 4 in accordance with the methods described herein.

Improvement of restorer

The *Brassica* inbred line BLR-038, Deposit Number NCIMB 41193 deposited on August 28, 2003, was crossed with high performing winter oilseed rape breeding lines, which are used as recurrent parents. In these crosses, the inbred line BLR-038 was used as female to

maintain the CMS cytoplasm. In thus obtained F1 plants were crossed with the recurrent parents to replace more of the genome of the inbred line BLR-038. Due to the CMS cytoplasm in every generation the presence or absence of the restorer gene could be detected by fertility scoring. In the F2 generation homozygous restorer plants were detected in the greenhouse by the described marker analysis and selfed. F3 descendants of the homozygous F2 plants were planted into the field to select only within the desired homozygous restorer plants. This helped to overcome a reduced amount of homozygous offsprings that were shown by the testcrosses. F3 plants are then selfed. Testcrosses were performed by using several CMS Ogura male sterile lines with a set of genetically different F4 or subsequent generation inbred plants as the female parents containing the BLR1 recombination event of the invention. The descendants were sown in the greenhouse and fertile and sterile plants were counted during flowering. Plants containing BLR1 recombination event can also be selected using the kit and method described herein.

Hybrid Development

FIG. 2 illustrates the conventional hybrid production scheme using CMS ogura and restorer line. As explained above, a male-sterile, female CMS A-line cannot self-pollinate, so it is maintained by crossing it with a maintainer B-line that is male fertile and genetically identical to the A-Line. The result of this cross is a male-sterile CMS line. The restorer R-line can be maintained by selfing. The restorer R-line is crossed with the male sterile CMS line to produce F1 seed produced on the A-line. The F1 seed are sold commercially for the production of F2 seed. The F2 seed of the invention has a low glucosinolate level as shown in Table 5. Table 5 shows the use of the *Brassica* inbred line BLR-038 to pollinate three different CMS inbred lines to produce three different hybrids. The GSL content of the F2 seed produced by the fertilized CMS plants showed substantially lower GSL content than a conventional Ogura restorer hybrid and are comparable to the desirable GSL levels of conventional non-restorer lines such as EXPRESS and SMART.

TABLE 5

	Oil %	GSL µmol
CMS female parent X BLR-038		
CMS line RNX 4801 X 01 21615-05 (BLR 038 Restorer)	40	13.9
CMS line RNX 4002 X 01 21615-08 (BLR 038 Restorer)	38	16.65
CMS line RNX 4901 X 01 21615-05(BLR 038 Restorer)	41.9	12.55
CMS line RNX 4801 X RNX 6001 (conventional Restorer)	41.3	29.7
EXPRESS (conventional line variety)	39.3	15.6
SMART (conventional line variety)	39.9	12.0

The foregoing invention has been described in detail by way of illustration and example for purposes of clarity and understanding. However, it will be obvious that certain changes and modifications such as single gene modifications and mutations, somaclonal variants, variant individuals selected from large populations of the plants of the instant inbred and the like may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

CLAIMS

What is claimed is:

1) A *Brassica* plant comprising a DNA fragment including a restorer gene, wherein said DNA fragment hybridizes to at least one marker of bin 2, but not to a marker of bin 3.

2) The *Brassica* plant according to claim 1, wherein bin 2 is comprised of the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2.

3) The *Brassica* plant according to claim 1, wherein bin 3 is comprised of the markers OPY17, OPN20, and E8M1-2.

4) The *Brassica* plant according to claim 2, wherein said markers are amplified in a polymerase chain reaction using primer pairs represented by 1159 and 1160; E2 and M4; E3 and M1; E4 and M14; E5 and M1; E5 and M4; E8 and M14, respectively.

5) The *Brassica* plant according to claim 3, wherein said markers are amplified in a polymerase chain reaction using the primer pairs represented by PR0004F and PR0004R; 1135 and 1136; and E8 and M1, respectively.

6) The *Brassica* plant according to claim 1, wherein said DNA fragment is the BLR1 recombination event.

7) The *Brassica* plant according to claim 1, wherein said plant is an inbred plant.

8) The *Brassica* plant according to claim 1, wherein said plant is a hybrid plant.

9) The *Brassica* plant according to claim 6, wherein said BLR1 recombination event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

10) A method of detecting a *Brassica* plant containing a restorer gene, comprising the steps of:

a) obtaining a sample from a *Brassica* plant;
b) detecting in said sample a DNA fragment that can be detected by a marker of bin 2, but not by a marker of bin 3.

11) The method of detecting a *Brassica* plant according to claim 10, further comprising selecting said *Brassica* plant, or a part thereof, containing said DNA fragment.

12) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of selfing said *Brassica* plant containing said DNA fragment.

13) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of crossing said *Brassica* plant with another *Brassica* plant.

14) The method of selecting a *Brassica* plant according to claim 10, wherein said DNA fragment comprises the BLR1 recombination event.

15) The method of selecting a *Brassica* plant according to claim 10, wherein said marker of bin 2 comprises E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

16) The method of selecting a *Brassica* plant according to claim 10, wherein said marker of bin 2 has partial homology to E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

17) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of detecting in said sample a DNA fragment obtainable by PCR amplification using primers 1159 and 1160, whereas said DNA fragment is not amplified by the primers PR0004F and PR0004R.

18) A combination of markers for detecting the presence of the BLR1 recombination event, comprising a marker of bin 2 and a marker of bin 3.

19) The combination of markers for detecting the presence of the BLR1 recombination event according to claim 18, wherein said marker of bin 2 comprises the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2 and wherein said marker of bin 3 comprises OPY17, OPN20, or E8M1, or a marker having partial homology to any one of these markers.

20) A method for screening a *Brassica* plant to determine whether it contains the BLR1 recombination event, comprising extracting DNA from said *Brassica* plant, subjecting the extraction to a polymerase chain amplification reaction in the presence of DNA fragments represented by primers 1159, 1160, PR0004F, and PR0004R, and determining the amplification of DNA fragments from the extracted DNA by primers 1159 and 1160 and lack of amplification of DNA fragments from extracted DNA that correspond to primers PR0004F and PR0004R.

21) A method for producing a fertile F1 hybrid *Brassica* plant comprising the steps of crossing a *Brassica* male fertile plant comprising the BLR1 recombination event with a *Brassica* CMS male sterile plant to produce F1 hybrid seed.

22) The method for producing a fertile F1 hybrid *Brassica* plant according to claim 21, comprising the additional step of planting said F1 hybrid seed.

23) The method for producing a fertile F1 hybrid *Brassica* plant according to claim 21, comprising the additional step of harvesting the F2 seed grown from the plant resulting from said F1 seed.

24) A hybrid F1 *Brassica* plant produced by the method of claim 21.

25) A *Brassica* plant comprising the BLR1 recombination event, wherein said event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

26) A method for producing a *Brassica* plant containing the BLR1 recombination event comprising the steps of obtaining a *Brassica* plant containing the BLR1 recombination event, crossing this plant with a another *Brassica* plant, obtaining hybrid seed produced by this cross, and planting said hybrid seed to produce a *Brassica* plant containing the BLR1 recombination event.

27) A kit for detecting the BLR1 recombination event comprising:
a) a first pair of primers that amplify a marker of bin 2; and
b) a second pair of primers that does not amplify a marker of bin 3.

28) A *Brassica* plant comprising the BLR1 recombination event.

29) The *Brassica* plant according to claim 28, wherein said BLR1 recombination event is obtainable from the Brassica inbred line BLR-038.

30) The *Brassica* plant according to claim 28, wherein said plant is a *Brassica napus*, *Brassica campestris*, *Brassica oleracea*, *Brassica nigra*, *Brassica carinata* or any other specie belonging to the *Brassicacea* family.

31) The *Brassica* plant according to claim 30, wherein said plant is a sexual or asexual recombination or clone of said species.

32) The *Brassica* plant according to claim 6, said plant comprising a glucosinolate level equal to or lower than the glucosinolate levels of double-low *Brassica* varieties.

IMPROVED FERTILITY RESTORATION FOR OGURA CYTOPLASMIC MALE STERILE *BRASSICA* AND METHOD

FIELD OF THE INVENTION

This invention relates to a novel fertility restorer locus for Ogura cytoplasmic male sterile *Brassica* plants.

BACKGROUND OF THE INVENTION

Oilseed rape (*Brassica napus*), also referred to as canola (annual spring type) or winter oilseed rape (biannual type), is derived from interspecific hybridization of *B. oleracea* and *B. campestris*. Breeding between *Brassica* species is common. Generally, winter-type rapeseed is grown in North Western Europe, whereas spring-types are grown in Canada, China, India, Australia and South America mainly.

Oilseed rape is becoming an increasingly important crop, valued for edible and industrial oil usage and for its seed meal rich in protein. Wide acceptance of rapeseed meal for animal nutrition is hampered by the presence in the seed of sulfur compounds called glucosinolates (GSLs). Glucosinolates are undesirable in Brassica seeds since they can lead to the production of antinutritional breakdown products upon enzymatic cleavage during oil extraction and digestion. Although the development of superior, edible oil had been achieved in the early 1970s through introduction of rapeseed varieties with less than 2% of erucic acid in percent of their total fatty acid profile (single zero quality), the continuing presence of glucosinolates in the high protein meal remained a major constraint to further market expansion.

At present the maximum threshold for GSL free rapeseed varieties set by European law is 25μmol total glucosinolate (GSL) per gram (g) of seed at 9% humidity (EU decree 2294/92). Doublelow spring canola varieties cultivated in Canada need to have GSL levels of less than 30μmoles of glucosinolates per gram of air-dried oil-free meal. The GSL levels of commonly cultivated double zero oilseed rape varieties in Europe and Canada varies significantly below the threshold levels at 60% of the official threshold level or even lower. At present, hybrid *Brassica* plants based on the Ogura hybrid system having seeds with low

GSL content express inferior agronomical traits such as lower seed yields, poor disease resistances and lodging susceptibility.

Hybrid cultivars are desired because of potentially higher seed yield due to heterosis. To produce hybrid *Brassica* plants, breeders use self-incompatible (SI), cytoplasmic male sterile (CMS), or nuclear male sterile (NMS) *Brassica* plants as the female parent. SI plants are not able to self pollinate due to their genetic constitution and CMS and NMS female plants are incapable of producing pollen. Thus, all these plants must be cross-pollinated by a male parent. A number of CMS systems are used for hybrid seed production of *Brassica*: Polima (*pol*), *nap*, *tournefortii*, Kosena, and Ogura (*ogu*). (See for example Ogura (1968) Mem. Fac. Agric. Kagoshima Univ. 6:39-78; Makaroff (1989 Journal of Biol. Chem. 264: 11706-11713; US Pat. No. 5,254,802.) The *ogu* system, thought to be the most useful, is based on the use of a male sterility determinant derived from *Raphanus sativus* cytoplasm. F1 seed produced from a cross between an Ogura CMS female *Brassica* plant and a normal male *Brassica* plant will be male sterile. In other words, plants grown from the F1 seed will not produce pollen. To produce a male fertile F1 generation plant, a restorer gene must be present in the male parent of the F1 hybrid.

A fertility restorer locus was transferred from *Raphanus sativus* to *Brassica* CMS plants by Institut National de la Recherche Agronomique (INRA) in Rennes, France (Pelletier et al., 1987) Proc 7th. Int. Rapeseed Conf., Poznan, Poland: 113-119. The restorer gene (Rf) originating from *Raphanus sativus* is described in WO92/05251 and in Delourme et al., (1991) Proc. 8th. Int. Rapeseed Conf. Saskatoon, Canada: 1506-1510. The original restorer inbreds and hybrids carrying this Rf gene express elevated glucosinolate levels and a decrease in seed set (Pellan-Delourme and Renard, 1988 Genome 30: 234-238, Delourme et al., 1994 Theor. Appl. Genet. 88: 741-748). In seed grown on Ogu Rf hybrid plants, the glucosinolate levels are elevated even when the female parent has reduced glucosinolate content. Recombination at the radish chromosomal region surrounding the Rf gene is suppressed in *Brassica* and therefore different recombination events in this region are difficult to obtain. The link between the Rf gene and the glucosinolate locus has been broken (WO98/27806). However, it is difficult to break the linkage between the glucosinolate gene and the restorer gene and still maintain line stability and superior combining ability for the production of high value commercial hybrid seed. Therefore, there is a need to develop a recombination event

that unlinks the restorer gene from the glucosinolate gene while maintaining a *Brassica* plant's ability to produce high value commercial hybrid seed.

SUMMARY OF THE INVENTION

The current invention provides a *Brassica* plant that comprises a recombination event resulting from a break between the restorer locus and the glucosinolate locus along a nucleic acid segment derived from the ogura *Raphanus sativus* and subsequent rejoining to produce a new nucleic acid segment, referred to herein as the BLR1 recombination event.

A *Brassica* plant of the present invention expresses fertility restoring resulting from expression of the *Raphanus sativus* restorer gene and a GSL content no higher than normal doublelow open pollinated varieties (varieties low in erucic acid in the oil and low in GSL in the solid meal remaining after oil extraction). The *Brassica* inbred line BLR-038, Deposit Number NCIMB-41193, is one example of a plant that contains the BLR1 recombination event of the invention. Using breeding techniques known to those skilled in the art and as briefly described herein, inbred line BLR-038 and other plants containing the BLR1 recombination event of the invention are crossed with male sterile inbreds to produce hybrids expressing low GSL content and superior agronomic traits. More generally, the present invention also includes transferring the BLR1 recombination event of the present invention from one *Brassica* plant to another. The present invention further includes the use of marker-assisted selection to select *Brassica* plants containing the BLR1 recombination event.

The invention includes a *Brassica* plant comprising a *Raphanus sativus* DNA fragment including a restorer gene, wherein said DNA fragment hybridizes to at least one marker of bin 2, but not to a marker of bin 3, and wherein the DNA fragment is the BLR1 recombination event of the present invention.

The present invention includes markers of bin 2 comprising E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2, and markers of bin 3 comprising OPY17, OPN20, and E8M1-2.

The present invention further includes the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2 and E8M14-2, which are amplified in a polymerase chain reaction using primer pairs represented by 1159 and 1160; E2 and M4; E3 and M1; E4 and M14; E5 and M1; and E8 and M14, respectively.

The present invention also includes the markers OPY17, OPN20, and E8M1-2, which are amplified in a polymerase chain reaction using the primer pairs represented by PR0004F and PR0004R; 1135 and 1136; and E8 and M1.

The invention includes the BLR1 recombination event, which is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

The present invention includes a method of detecting a *Brassica* plant containing a restorer gene derived from *Raphanus sativus*, comprising the steps of: obtaining a plant sample from a *Brassica* plant, detecting in the sample a DNA fragment that can be detected by a marker of bin 2, but not by a marker of bin 3. The method further includes selecting the *Brassica* plant, or a part thereof, containing the DNA fragment, and also selfing the *Brassica* plant containing the DNA fragment. Preferably, the DNA fragment comprises the BLR1 recombination event.

The present invention further includes a method of detecting a *Brassica* plant, wherein the marker of bin 2 comprises E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

The invention includes a method of detecting a *Brassica* plant, wherein the marker of bin 2 has partial homology to E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

The method of the invention includes the step of detecting in a plant sample a DNA fragment obtainable by PCR amplification using primers 1159 and 1160, whereas the DNA fragment is not amplified by the primers PR0004F and PR0004R.

The present invention also includes a combination of markers for detecting the presence of the BLR1 recombination event, comprising a marker of bin 2 and a marker of bin 3.

The present invention further includes a marker of bin 2 comprising E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2 and a marker of bin 3 comprising OPY17, OPN20, and E8M1, or a marker having partial homology to any one of these markers.

The present invention also includes a method for screening a *Brassica* plant to determine whether it contains the BLR1 recombination event, comprising extracting DNA from the *Brassica* plant, subjecting the *Brassica* plant extraction to a polymerase chain amplification

reaction in the presence of primers 1159, 1160, PR0004F, PR0004R, and determining the amplification of DNA fragments from the extracted DNA by primers 1159 and 1160 and lack of amplification of DNA fragments from extracted DNA by primers PR0004F and PR0004R, thereby indicating the presence of the BLR1 recombination event.

The present invention also includes a method for producing a fertile F1 hybrid *Brassica* plant comprising the steps of crossing a Brassica male fertile plant comprising the BLR1 recombination event with a *Brassica* CMS male sterile plant to produce F1 fertile seed, further comprising the step of planting said F1 hybrid seed, and further comprising the step of harvesting the F2 seed grown from the plant resulting from said F1 seed, and includes F1 hybrid *Brassica* plants developed by this method.

The present invention also includes a *Brassica* plant comprising the BLR1 recombination event, wherein said event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

The present invention further includes a method of introgressing the BLR1 recombination event comprising the steps of obtaining a *Brassica* plant containing the BLR1 recombination event, crossing this plant with another *Brassica* plant, producing hybrid seed and selecting hybrid seed containing the BLR1 recombination event.

The present invention includes a kit and method that incorporate one or more of markers falling within bin 2 and one or more markers falling within bin 3 to detect the presence of the BLR1 recombination event in a plant or a plant part. According to the invention, plant material that contains the BLR1 recombination event hybridizes to bin 2 markers, but not to markers of bin 3.

BRIEF DESCRIPTION OF THE FIGURES

FIG 1 is a photocopy of an agarose gel illustrating amplification products that are generated and analyzed by the multiplex PCR kit and method of the present invention. Lane 1: random maintainer genotype rr; 2: Pioneer 209002; 3: BLR 038; 4: Pioneer 97838, 5: Pioneer 97839; 6: Pioneer 209001; 7: original *Ogura* restorer Rr, 8: Lutin (INRA) a *Raphanus sativus* restorer gene.

FIG. 2 is a schematic depiction of the F1 hybrid production scheme for CMS *Brassica* plants.

DETAILED DESCRIPTION OF THE INVENTION

The current invention provides a *Brassica* plant comprising a unique recombination event, referred to herein as the BLR1 recombination event, due to a break at a position along the radish nucleic acid segment between the restorer locus and the glucosinolate locus. *Brassica* plants of the present invention express fertility restoring resulting from expression of the *Raphanus sativus* restorer gene and a GSL content no higher than normal double low open pollinated varieties. The *Brassica* inbred line BLR-038, Deposit Number NCIMB-41193, is one example of a plant that contains the BLR1 recombination event. Using breeding techniques known to those skilled in the art and as briefly described herein, inbred line BLR-038 and other inbred lines containing the BLR1 recombination event are crossed with male sterile inbreds to produce hybrids expressing low GSL content and superior agronomic traits. More generally, the present invention further includes transferring the BLR1 recombination event of the present invention from one *Brassica* plant to another. A further aspect of the invention is a kit and method including markers and the use of markers of specified bins to select *Brassica* plants that contain the BLR1 recombination event.

Breeding History of the *Brassica* inbred line BLR038 and GSL characterization

Table 1 describes the breeding history of plants of the present invention containing the BLR1 recombination event, which is a recombination of the ogura *Raphanus sativus* restorer locus. In year 1992, the CMS inbred line R30915 was crossed with the male inbred line R40 containing the restorer gene of INRA, to produce F1 hybrids. R40 is a generation F6 offspring produced via selfings from the original cross (Fu 58.Darmor B1F1 x Rest. Darmor B1F1) x Bienvenu. F1 hybrids from the cross R30915 x R40 with the CMS-restorer gene were selected based on male fertility, which was determined at flowering. The F1 hybrid plants (92HR013) were crossed with a non-CMS, non-restorer double zero quality breeding line 93B-1-3. In 1994, seeds of fertile plants resulting from the cross with 93B-1-3 were grown and the resulting CMS restorer plants were crossed with the double low quality breeding line 92/19047. The lines resulting from this cross were selfed several times from 1995 through 2002 as shown in Table 1. In all plots, segregation of male fertility was

observed, meaning that all plots contained heterozygous and homozygous maintainer and restorer plants. Because all crosses were initially made in the Ogura CMS cytoplasm and this cytoplasm was maintained in all future generations the maintainer genotypes turned out to be male sterile. Plants were selfed using plastic bags to cover the inflorescence before flowering. The bag was maintained over the plant during the whole flowering period to avoid cross-pollination.

The GSL content of the *Brassica* seeds was monitored throughout the development of inbred line BLR-038. Glucosinolate content is given in µmol/g of seed at 9% humidity. The glucosinolate analysis was performed using the near-infrared reflectance spectroscopy. Using this method, it is possible to analyze samples of undestroyed *Brassica* seed on their quality components oil, protein and glucosinolate. The analyses were performed on a FOSS NIR Systems Model 5000-c. Glucosinolate analysis is described in P. Williams and D. Sobering, (1992) In: Hildrum K., Isaksson T., Naes T. and Tandberg A. (eds.) Near Infra-red Spectroscopy. Bridging the gap between Data Analysis and NIR Applications. Horwood Chichester, UK: 41-446

In 1999, one plant of the F6 generation, 22044-3, had a GSL content of 17.3 µmol/g seed, while the seed of its sister plants had a GSL content between 22.5-23.8 µmol/g. Plant 22044-3 was selfed resulting in plants of the F7 generation. Seed of the 6797-2 plant had a GSL content of 11.4 µmol/g, while its sister plants had a GSL content from 24.6-25.7 umol/g. The plant resulting from growing the seed of 6797-2 was selfed. In 2001 at F8, no single plant resulting from this selfing had seed with a GSL content above 14.3 umol/g. The seed of plant 21615-7 had a GSL content of only 7.0 umol/g. The average expression of seed from plants in plot 21615 was 10.7 umol/g, which is at least 7 µmol lower than the lowest other reference restorer lines grown simultaneously in the same experimental field trial in Germany and more than 5 µmol below the standard plots of the non-restorer varieties Express and Laser. At the F9 generation, BLR-038 was produced by selfing of homozygous descendants of 21615-5.

TABLE 1

Pollination	Year	Generation	PLOT	Cross	Plot µmol/g Seed	Single plant No. (GSL µmol/g seed at 9% H_2O)
cross	1992		92HR013	R30195 (CMS B6 021) x R40		
cross	1993		93HR141	92HR013 x 93B-1-3		
cross	1994	F1	94HR233	93HR141 X 92/19047		
selfing	1995	F2	21614			9
selfing	1996	F3	21969			3
selfing	1997	F4	22446			8
selfing	1998	F5	22590			1
selfing	1999	F6	22044	GSL content of sister plants was 22.5-23.8		3(17.3)
selfing	2000	F7	6797	GSL content of sister plants was 24.6-25.7		2 (11.4)
selfing	2001	F8	21615	No single plants with GSL content above 14.3µmol were observed	10.7	1 (10.3), 2 (9.4), 4 (14.1), 5 (8.6), 6 (9.4), 7 (7.0), 8 (14.3)
selfing	2002	F9	21615-5			BLR-038

Table 2 shows the segregation ratio for several of the single plants of plot 01-21615. The Rf pollinator plants (21615-01, 21615-05, 21615-06, 21615-08) are homozygous for the Rf gene (RfRf). F1 hybrids were produced from the cross of the homozygous Rf pollinator and CMS female lines. These crosses show a transmission of male fertility of approximately 100%.

TABLE 2

	Homozygous Pollinator							
	F1 hybrids				Selfing of Pollinator			
Origin 2001 (F8)	plants	male sterile	male fertile	ratio fertile plants; expected 100%	plants	male sterile	male fertile	ratio fertile plants; expected 100%
01 21615-01	12	0	12	100.0%	9	0	9	100.0%
01 21615-05	39	1	38	97.4%	14	1	13	92.9%
01 21615-06	16	0	16	100.0%	12	0	12	100.0%
01 21615-08	11	1	10	90.9%	9	0	9	100.0%
SUM	78	2	76	97.4%	44	2	43	97.7%

Characterization of *Brassica* inbred line BLR-038 by means of AFLP analysis

A population consisting of 25 individuals segregating for the original Ogura restorer translocation was genotyped using a co-dominant PCR assay consisting of two proprietary SCAR markers derived from RAPD marker OPY17 that are in coupling or in repulsion phase to the restoration locus. Homozygous recessive (rf/rf) plants and restorer (RfRf and Rfrf) plants were bulked separately and used for the identification of AFLP markers putatively linked to the Rf gene. Such markers allowed for the comparison of BLR-038 to Pioneer hybrids 209002, 97839, 97838, 209001, and to the SERASEM hybrid Lutin containing the restorer locus released by INRA. AFLP analysis was performed essentially as described by Vos et al. (1995) Nucleic Acids Research 23(21): 4407-4414.

First, 500 ng DNA for each sample BLR-038, 209002, 97839, 97838, 209001, and the hybrid Lutin, was digested in 40μl of 1×TA-buffer (10 mM Tris-acetate, 10 mM MgAc, 50 mM KAc, 1 mM DTT, 2 μg BSA and 5 u each of *Eco*RI and *Tru*1I (MBI Fermentas, Lithuania). *Eco*RI is in the following referred to as E, and *Tru*1I, an isoschizomer of *Mse*I, is referred to as M. The E and M adaptors are represented by the following sequences:

EcoRI-adaptor:	5'-CTCGTAGACTGCGTACC	SEQ ID NO: 21
	CATCTGACGCATGGTTAA-5'	SEQ ID NO: 22
MseI-adaptor:	5'-GACGATGAGTCCTGAG	SEQ ID NO: 23
	TACTCAGGACTCAT-5'	SEQ ID NO: 24

Following digestion, 10 μl of ligation solution containing 1×Ligation buffer (40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 1 u T4 DNA ligase, 0.1 μM E-adapter and 1.0 μM M-adapter, sequences as described by Vos et al. (1995), was added directly to the DNA digest, incubated, and subsequently diluted 10-fold in 1×TE-buffer. To increase the amount of template DNA, the diluted ligation reactions were preamplified with primers having one additional and selective nucleotide each, i.e. E+1 and M+1, The primers used for the pre-amplification reaction consist of the same sequence as the adapters except for a one nucleotide extension at their 3' end. Primer E+A hybridizes to the EcoRI adapter and carries an additional A, the primer M+C hybridizes to the MseI adapter and carries an additional C. The reaction solution of 20 μl contained 5 μl of template DNA (10-fold diluted ligation reaction), 1×PCR-buffer II (10 mM Tris-HCl,pH 8.3), 50 mM KCl, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.4 u *Taq* polymerase and 0.3 μM each of (E+A)-primer and (M+C)-primer. The pre-ampflication reactions were performed in either Perkin-Elmer/Cetus 9600 or MJ Research PTC-100 thermocyclers using the following temperature profile: 20 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C.

Prior to selective amplification, (E+3)-primers were end-labelled in a solution containing 1×kinase buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 1.7 μM (E+3)-primer (DNA Technologies), 0.2 u/μl T4 polynucleotide kinase and 2 μCi/μl μ-^{33}P[ATP]. Selective amplification was performed using the following temperature profile: 12 cycles of 30 s at 94°C, 30 s at 65°C ramping 0.7°C/cycle to 56°C, 60 s at 72°C, followed by 23 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C. The reaction solution of 20 μl contained 5 μl pre-amplified template DNA, 0.5 μl labelled (E+3)-primer, 1×PCR-buffer II (Advanced Biotechnologies), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μM (M+3)-primer (DNA Technologies), and 0.4 u of *Taq* polymerase. After amplification 20 μl of formamide loading buffer (98% formamide, 10 mM EDTA, 0.1% each of xylene cyanol and bromophenol blue) was added and the samples were denatured at 95°C

for 3 min. Amplified fragments were separated on 5% polyacryamide gels consisting of 19:1 Acrylamide/Bis solution, 1×TBE-buffer, 0.10% TEMED and 0.03% APS. Custom-made gel apparatuses for 35 cm gels (CBS Scientific Co., USA) were used in all analyses. Gels were pre-run at 110 W for 30 min before loading of 3 µl sample and run at 110W for 3 h. Following electrophoresis, gels were transferred to 3MM-paper, dried on a gel dryer over night at 80°C, and exposed to film for 1-2 days.

All E+3 primers (24 nt in length) as shown in Table 3 (SEQ. ID No. 25 to 37) and the sequence listing carry an A at position 22 and all M+3 primers (21 nt in length) a C at position 19, which correspond to the extensions on the pre-amplification primers. The extensions at the pre-amplification primers are random and are added for the purpose of reducing the complexity of the template. Rather than amplifying the whole genome, only a fraction is amplified that subsequently is used as template in the final amplification using the E+3 and M+3 primers. The E+A and M+C pre-amplification primers are identical to the E+3 and M+3 primers respectively, but two nucleotides shorter. It is understood that one skilled in the art can develop additional primers by generating additional randomly generated extensions to the adaptors M and E. Some of these new primers would amplify additional nucleic acid segments or markers located along the nucleic acid segment derived from ogura *Raphanus sativus* and would be categorized within one of the four bins. Those skilled in the art would recognize that these additional primers and markers fall within the scope of the claimed invention.

In total 48 primer combinations were screened, including the 7 primer pairs that were shown to deliver polymorphic bands in patent application WO98/56948. Only bands that were present in the Ogura Rf bulk but absent in the homozygous recessive bulk (rf/rf) were taken into consideration for the comparison of the *Brassica* inbred line BLR-038 to the hybrids released by Pioneer and INRA.

Table 3 shows all AFLP markers that revealed polymorphism between the bulk for the Ogura Rf translocation and the homozygous recessive (rfrf) bulk. The markers are binned according to their amplification profile across the various plant materials. The results are represented in a schematic manner in Table 4, which reveals the four different classes of markers. Presence of a band is indicated with '1', its absence with '0'. A bin refers to a set of markers grouped according to their location along a nucleic acid segment. AFLP markers

E5M16-1, E5M4-3, E6M3-2, and E8M14-1 are of bin 1, wherein these markers are amplified in all samples Lutin, P209001, P97838, P97839, BLR-038, and P209002. ALFP markers E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, E8M14-2 are of bin 2, wherein bin 2 markers amplify Lutin, P209001, P97838, P97839, BLR-038, but not P209002. The AFLP marker E8M1-2 is of bin 3, wherein bin 3 markers amplify Lutin, P209001, P97838, P97839, but not BLR-038, and P209002. The AFLP markers E2M13-1, E2M14-1, E3M12-1, and E6M3-1 are of bin 4, wherein bin 4 markers amplify Lutin and P209001, but not P97838, P97839, BLR-038, and P209002.

Characterization of the *Brassica* inbred line BLR-038 using SCAR markers

Primer pairs were designed to the nucleotide sequences of the amplification products for the RAPD, AFLP and SCAR markers in coupling phase with the Ogura restorer gene as disclosed in patent application CA2,206,673: OPC2 (Seq ID No. 2 and 7), OPN20 (Seq ID No. 3 and 8), OPF10 (Seq ID No. 4 and 10), OPH3 (Seq ID No. 9), OPH15 (Seq ID No. 11), E36xM48AIII ((Seq ID No. 12), E35xM62AV (Seq ID No. 13), E33xM47A1 (Seq ID No. 14), and E38xM60A1 (Seq ID No. 15). In addition to these markers, primers were designed to the nucleotide sequence of RAPD marker OPH11 that was shown to be associated to fertility restoration in *Raphanus* where the Ogura locus originates (Accession number AB051636). The sequences of all primers assayed as well as the size of the expected amplification products are listed in Table 3. The primer combinations including the proprietary SCAR marker derived from RAPD marker OPY17, were used to analyze the original Ogura translocation, BLR038, Pioneer hybrids 209002, 97839, 97838, 209001, and the hybrid Lutin using a standard PCR protocol. After PCR, the amplification products were visualized by means of agarose gel electrophoresis. Referring to Table 4, the SCAR markers OPF10, OPC2 AND E35M62 are markers of Bin 1. Markers that fall within Bin 1, as discussed above, are characterized as amplifying the samples Lutin, P209001, P97838, P97839, BLR-038, and P209002. The SCAR marker E33M47 is of bin 2. Bin 2 markers are characterized as amplifying the samples Lutin, P209001, P97838, P97839, BLR-038, but not P209002. The two SCAR markers, OPY17 and OPN20 of Bin 3, are characterized by amplifying the samples Lutin, P209001, P97838, P97839, but not BLR-038, and P209002. Bin 4 SCAR markers, such as OPH15 and E36M48, amplify Lutin and P209001, but not P97838, P97839, BLR-038, and P209002.

TABLE 3

Marker Locus	Primer Pair	Sequence	Product Size	Origin of sequence
SCAR markers and primers				
OPC2	1127 (SEQ ID NO: 1) 1128 (SEQ ID NO: 2)	ggggaaggaaggaaggactc tcaggttcacacagcagcata	677 bp	CA 2,206,673
OPN20	1135 (SEQ ID NO: 3) 1136 (SEQ ID NO: 4)	ataggttcctggcagagatg atagcagtcagaaaccgctc	630 bp	CA 2,206,673
OPF10	1137 (SEQ ID NO: 5) 1138 (SEQ ID NO: 6)	ctgatgaatctcggtgagac ccgtatgccttggttatctc	760 bp	CA 2,206,673
OPH15	1218 (SEQ ID NO: 7) 1219 (SEQ ID NO: 8)	tctgtaaatcctttccaccc aaaaaagcacccgagaatct	601 bp	CA 2,206,673
E36M48	1222 (SEQ ID NO: 9) 1223 (SEQ ID NO: 10)	gcgtgatgatctgttgagaa ggatttgtgggattggaaa	251 bp	CA 2,206,673
E35M62	1224 (SEQ ID NO: 11) 1225 (SEQ ID NO: 12)	gaggttcaggaatgctgttt gctcctgttagtgactcttca	201 bp	CA 2,206,673
E33M47	1159 (SEQ ID NO: 13) 1160 (SEQ ID NO: 14)	taacaaaatagagggagaggatg caagattatagctacctaacagg	140 bp	CA 2,206,673
Gene 16	16-1 (SEQ ID NO: 15) 16-2 (SEQ ID NO: 16)	tgttcagcatttagtttcgccc ttgttcagttccaccaccagcc	471 bp	WO 03/006622
Gene 26	26-1 (SEQ ID NO: 17) 26-2 (SEQ ID NO: 18)	gctcacctcatccatcttcctcag ctcgtcctttaccttctgtggttg	530 bp	WO 03/006622
OPY17	PR0004F (SEQ ID NO: 19) PR0004R (SEQ ID NO: 20)	acgtggtgaggacatgccctttctg ctggtgtattctacctcatcattaaa	300 bp	Syngenta
AFLP markers and primers				
E2M4	E2 (SEQ ID NO: 25) M4 (SEQ ID NO: 26)	ctcgtagactgcgtaccaattaac gacgatgagtcctgagtacat		
E2M13	E2 M13 (SEQ ID NO: 27)	 gacgatgagtcctgagtacta		
E2M14	E2 M14 (SEQ ID NO: 28)	 gacgatgagtcctgagtactc		

E3M1	E3 (SEQ ID NO: 29) M1 (SEQ ID NO: 30)	ctcgtagactgcgtaccaattaag gacgatgagtcctgagtacaa		
E3M12	E3 M12 (SEQ ID NO: 31)	 gacgatgagtcctgagtacgt		
E4M14	E4 (SEQ ID NO: 32) M14	ctcgtagactgcgtaccaattaat		
E5M1	E5 (SEQ ID NO: 33) M1	ctcgtagactgcgtaccaattaca		
E5M4	E5 M4			
E5M16	E5 M16 (SEQ ID NO: 34)	 gacgatgagtcctgagtactt		
E6M3	E6 (SEQ ID NO: 35) M3 (SEQ ID NO: 36)	ctcgtagactgcgtaccaattacc gacgatgagtcctgagtacag		
E8M1	E8 (SEQ ID NO: 37) M1	ctcgtagactgcgtaccaattact		
E8M14	E8 M14			

TABLE 4

Marker	Type	Rr	Rr	Lutin	P 209001	P 97838	P 97839	BLR-038	P 209002	
		1	2	8	6	4	5	3	7	
OPF10	**SCAR**	1	0	1	1	1	1	1	1	
OPC2	**SCAR**	1	0	1	1	1	1	1	1	
E35M62	**SCAR**	1	0	1	1	1	1	1	1	
E5M16-1	**AFLP**	1	0	1	1	1	1	1	1	BIN 1
E5M4-3	**AFLP**	1	0	1	1	1	1	1	1	
E6M3-2	**AFLP**	1	0	1	1	1	1	1	1	
E8M14-1	**AFLP**	1	0	1	1	1	1	1	1	
E33M47	**SCAR**	1	0	1	1	1	1	1	0	
E2M4-1	**AFLP**	1	0	1	1	1	1	1	0	
E3M1-1	**AFLP**	1	0	1	1	1	1	1	0	
E4M14-1	**AFLP**	1	0	1	1	1	1	1	0	BIN 2
E5M1-2	**AFLP**	1	0	1	1	1	1	1	0	
E5M4-2	**AFLP**	1	0	1	1	1	1	1	0	
E8M14-2	**AFLP**	1	0	1	1	1	1	1	0	
OPY17	**SCAR**	1	0	1	1	1	1	0	0	
OPN20	**SCAR**	1	0	1	1	1	1	0	0	BIN 3
E8M1-2	**AFLP**	1	0	1	1	1	1	0	0	
OPH15	**SCAR**	1	0	1	1	0	0	0	0	
E36M48	**SCAR**	1	0	1	1	0	0	0	0	
E2M13-1	**AFLP**	1	0	1	1	0	0	0	0	BIN 4
E2M14-1	**AFLP**	1	0	1	1	0	0	0	0	
E3M12-1	**AFLP**	1	0	1	1	0	0	0	0	
E6M3-1	**AFLP**	1	0	1	1	0	0	0	0	

Kit and method for detecting the BLR1 recombination event

Total DNA is isolated from approximately 1 cm^2 of *Brassica* leaf tissue by using the Wizard® Magnetic 96 DNA Plant System (Promega). In one embodiment, the Multiplex PCR kit and method of the present invention detects the presence or absence of PCR amplification products corresponding to OPY17 (Bin 3) and E33M47 (Bin 2).

The four primers PR0004F, PR0004R, 1159 and 1160 (Table 4) are added to a reaction mixture at a concentration of 7.5 pmol each. Except for the multiplex nature, the composition of the PCR reaction is standard in the art, using Platinum Taq polymerase from Invitrogen. Amplification conditions are as follows: 5 minutes of initial denaturation at 94°C were followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 90 seconds at 72°C. PCR amplification products were separated on 2.0 % agarose gels.

Fig. 1 illustrates the results of the PCR reaction, wherein the presence of the BLR1 recombination event is established when the primers amplify the 140 bp product that corresponds to E33M47, but does not amplify the 300 bp product that corresponds to OPY17. Fig. 1 also shows that the PCR reaction amplified both OPY17 and E33M47 for the original *Ogura* restorer translocation fragment as well as the derived recombination events Pioneer 97838, 97839, 209001, and the Lutin event from INRA. Pioneer recombination event 209002 on the other hand, shows neither the E33M47, nor the OPY17 amplification product. These results demonstrate that primers that selectively amplify markers from Bin 2 and 3, such as E33M47 and OPY17, are successfully used in a single multiplex PCR assay to distinguish and identify the BLR1 recombination event in plant material.

It is understood that the kit and method of the present invention incorporate one or more of markers falling within Bin 3 and one or more markers falling within Bin 2 to detect the presence of the BLR1 recombination event in plants. It is within the scope of the claimed invention to develop and use additional markers that fall within one of the bins 1, 2, 3, or 4 in accordance with the methods described herein.

Improvement of restorer

The *Brassica* inbred line BLR-038, Deposit Number NCIMB 41193 deposited on August 28, 2003, was crossed with high performing winter oilseed rape breeding lines, which are used as recurrent parents. In these crosses, the inbred line BLR-038 was used as female to

maintain the CMS cytoplasm. In thus obtained F1 plants were crossed with the recurrent parents to replace more of the genome of the inbred line BLR-038. Due to the CMS cytoplasm in every generation the presence or absence of the restorer gene could be detected by fertility scoring. In the F2 generation homozygous restorer plants were detected in the greenhouse by the described marker analysis and selfed. F3 descendants of the homozygous F2 plants were planted into the field to select only within the desired homozygous restorer plants. This helped to overcome a reduced amount of homozygous offsprings that were shown by the testcrosses. F3 plants are then selfed. Testcrosses were performed by using several CMS Ogura male sterile lines with a set of genetically different F4 or subsequent generation inbred plants as the female parents containing the BLR1 recombination event of the invention. The descendants were sown in the greenhouse and fertile and sterile plants were counted during flowering. Plants containing BLR1 recombination event can also be selected using the kit and method described herein.

Hybrid Development

FIG. 2 illustrates the conventional hybrid production scheme using CMS ogura and restorer line. As explained above, a male-sterile, female CMS A-line cannot self-pollinate, so it is maintained by crossing it with a maintainer B-line that is male fertile and genetically identical to the A-Line. The result of this cross is a male-sterile CMS line. The restorer R-line can be maintained by selfing. The restorer R-line is crossed with the male sterile CMS line to produce F1 seed produced on the A-line. The F1 seed are sold commercially for the production of F2 seed. The F2 seed of the invention has a low glucosinolate level as shown in Table 5. Table 5 shows the use of the *Brassica* inbred line BLR-038 to pollinate three different CMS inbred lines to produce three different hybrids. The GSL content of the F2 seed produced by the fertilized CMS plants showed substantially lower GSL content than a conventional Ogura restorer hybrid and are comparable to the desirable GSL levels of conventional non-restorer lines such as EXPRESS and SMART.

TABLE 5

	Oil %	GSL μmol
CMS female parent X BLR-038		
CMS line RNX 4801 X 01 21615-05 (BLR 038 Restorer)	40	13.9
CMS line RNX 4002 X 01 21615-08 (BLR 038 Restorer)	38	16.65
CMS line RNX 4901 X 01 21615-05(BLR 038 Restorer)	41.9	12.55
CMS line RNX 4801 X RNX 6001 (conventional Restorer)	41.3	29.7
EXPRESS (conventional line variety)	39.3	15.6
SMART (conventional line variety)	39.9	12.0

The foregoing invention has been described in detail by way of illustration and example for purposes of clarity and understanding. However, it will be obvious that certain changes and modifications such as single gene modifications and mutations, somaclonal variants, variant individuals selected from large populations of the plants of the instant inbred and the like may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

CLAIMS

What is claimed is:

1) A *Brassica* plant comprising a DNA fragment including a restorer gene, wherein said DNA fragment hybridizes to at least one marker of bin 2, but not to a marker of bin 3.

2) The *Brassica* plant according to claim 1, wherein bin 2 is comprised of the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, and E8M14-2.

3) The *Brassica* plant according to claim 1, wherein bin 3 is comprised of the markers OPY17, OPN20, and E8M1-2.

4) The *Brassica* plant according to claim 2, wherein said markers are amplified in a polymerase chain reaction using primer pairs represented by 1159 and 1160; E2 and M4; E3 and M1; E4 and M14; E5 and M1; E5 and M4; E8 and M14, respectively.

5) The *Brassica* plant according to claim 3, wherein said markers are amplified in a polymerase chain reaction using the primer pairs represented by PR0004F and PR0004R; 1135 and 1136; and E8 and M1, respectively.

6) The *Brassica* plant according to claim 1, wherein said DNA fragment is the BLR1 recombination event.

7) The *Brassica* plant according to claim 1, wherein said plant is an inbred plant.

8) The *Brassica* plant according to claim 1, wherein said plant is a hybrid plant.

9) The *Brassica* plant according to claim 6, wherein said BLR1 recombination event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

10) A method of detecting a *Brassica* plant containing a restorer gene, comprising the steps of:

a) obtaining a sample from a *Brassica* plant;
b) detecting in said sample a DNA fragment that can be detected by a marker of bin 2, but not by a marker of bin 3.

11) The method of detecting a *Brassica* plant according to claim 10, further comprising selecting said *Brassica* plant, or a part thereof, containing said DNA fragment.

12) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of selfing said *Brassica* plant containing said DNA fragment.

13) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of crossing said *Brassica* plant with another *Brassica* plant.

14) The method of selecting a *Brassica* plant according to claim 10, wherein said DNA fragment comprises the BLR1 recombination event.

15) The method of selecting a *Brassica* plant according to claim 10, wherein said marker of bin 2 comprises E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

16) The method of selecting a *Brassica* plant according to claim 10, wherein said marker of bin 2 has partial homology to E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2.

17) The method of detecting a *Brassica* plant according to claim 10, further comprising the step of detecting in said sample a DNA fragment obtainable by PCR amplification using primers 1159 and 1160, whereas said DNA fragment is not amplified by the primers PR0004F and PR0004R.

18) A combination of markers for detecting the presence of the BLR1 recombination event, comprising a marker of bin 2 and a marker of bin 3.

19) The combination of markers for detecting the presence of the BLR1 recombination event according to claim 18, wherein said marker of bin 2 comprises the markers E33M47, E2M4-1, E3M1-1, E4M14-1, E5M1-2, E5M4-2, or E8M14-2 and wherein said marker of bin 3 comprises OPY17, OPN20, or E8M1, or a marker having partial homology to any one of these markers.

20) A method for screening a *Brassica* plant to determine whether it contains the BLR1 recombination event, comprising extracting DNA from said *Brassica* plant, subjecting the extraction to a polymerase chain amplification reaction in the presence of DNA fragments represented by primers 1159, 1160, PR0004F, and PR0004R, and determining the amplification of DNA fragments from the extracted DNA by primers 1159 and 1160 and lack of amplification of DNA fragments from extracted DNA that correspond to primers PR0004F and PR0004R.

21) A method for producing a fertile F1 hybrid *Brassica* plant comprising the steps of crossing a *Brassica* male fertile plant comprising the BLR1 recombination event with a *Brassica* CMS male sterile plant to produce F1 hybrid seed.

22) The method for producing a fertile F1 hybrid *Brassica* plant according to claim 21, comprising the additional step of planting said F1 hybrid seed.

23) The method for producing a fertile F1 hybrid *Brassica* plant according to claim 21, comprising the additional step of harvesting the F2 seed grown from the plant resulting from said F1 seed.

24) A hybrid F1 *Brassica* plant produced by the method of claim 21.

25) A *Brassica* plant comprising the BLR1 recombination event, wherein said event is obtainable from the *Brassica* inbred line BLR-038, a sample of the seed of inbred line BLR-038 having been deposited with NCIMB under accession number NCIMB 41193.

26) A method for producing a *Brassica* plant containing the BLR1 recombination event comprising the steps of obtaining a *Brassica* plant containing the BLR1 recombination event, crossing this plant with a another *Brassica* plant, obtaining hybrid seed produced by this cross, and planting said hybrid seed to produce a *Brassica* plant containing the BLR1 recombination event.

27) A kit for detecting the BLR1 recombination event comprising:

a) a first pair of primers that amplify a marker of bin 2; and

b) a second pair of primers that does not amplify a marker of bin 3.

28) A *Brassica* plant comprising the BLR1 recombination event.

29) The *Brassica* plant according to claim 28, wherein said BLR1 recombination event is obtainable from the Brassica inbred line BLR-038.

30) The *Brassica* plant according to claim 28, wherein said plant is a *Brassica napus, Brassica campestris, Brassica oleracea, Brassica nigra, Brassica carinata* or any other specie belonging to the *Brassicacea* family.

31) The *Brassica* plant according to claim 30, wherein said plant is a sexual or asexual recombination or clone of said species.

32) The *Brassica* plant according to claim 6, said plant comprising a glucosinolate level equal to or lower than the glucosinolate levels of double-low *Brassica* varieties.

ABSTRACT

A *Brassica* plant comprising a unique recombination event resulting from a break at a position along a nucleic acid segment derived from ogura *Raphanus sativus* between the restorer locus and the glucosinolate locus and subsequent rejoining to produce a new recombination event, BLR1. The BLR1 recombination event expresses fertility restoring resulting from expression of the restorer gene derived from *Raphanus sativus* and a GSL content no higher than normal double low open pollinated varieties. The *Brassica* inbred line BLR-038, Deposit Number NCIMB-41193, is one example of a plant that contains the BLR1 recombination event. The BLR1 recombination event is introgressed into different *Brassica* genetic backgrounds using breeding techniques known to those skilled in the art. For example, the *Brassica* inbred line BLR-038 or another *Brassica* plant containing the BLR1 recombination event may be crossed with male sterile inbreds to produce hybrids expressing low GSL content and superior agronomic traits.

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Stiewe, Gunther
Brummermann, Katja
Pleines, Stephan

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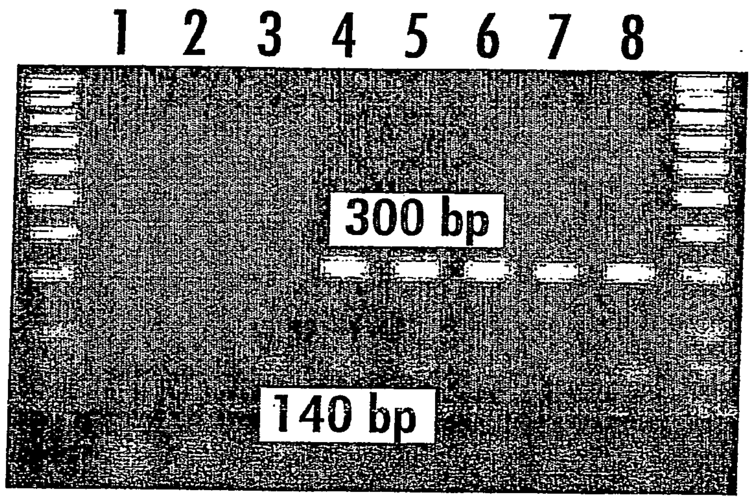
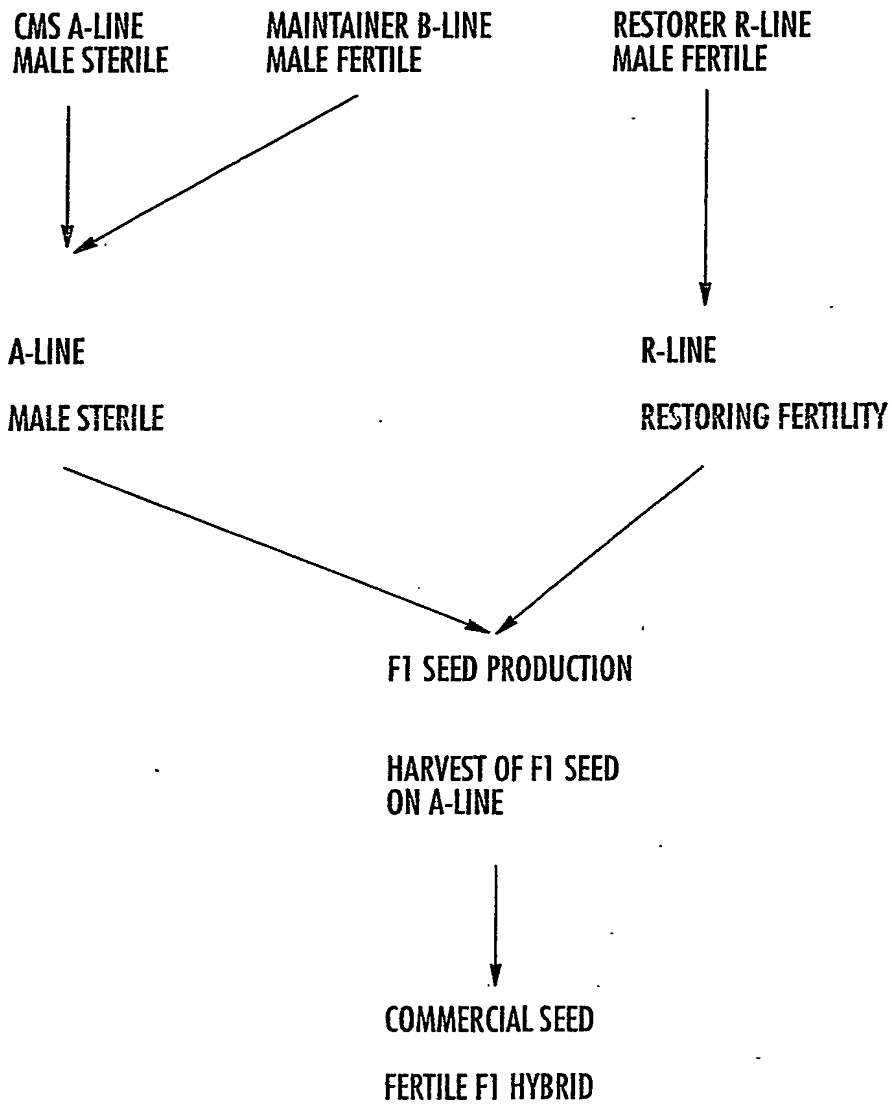


FIG. 1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RESTORING FERTILITY
F1 SEED PRODUCTION
HARVEST OF F1 SEED
ON A-LINE
COMMERCIAL SEED
FERTILE F1 HYBRID

FIG. 2

PCT/EP2005/000877

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